DESCRIPTION

Epilepsy Model Animal (chrna4:S284L)

Technical Field

The invention of this patent application relates to an epilepsy model animal having gene abnormality homologous to human autosomal dominant nocturnal frontal lobe epilepsy and developing spontaneous epileptic seizure during sleep.

Background Art

Epilepsy is a chronic brain disorder characterized by episodic seizures caused by excessive brain cell firing. The excessive brain cell firing is a result of various etiologic factors, and, accordingly, progression and prognosis of the seizure can vary greatly depending on the type of epilepsy. Therefore, accurate diagnosis and appropriate procedure are required for treating epilepsy.

An epilepsy model animal has been used as means for developing and progressing diagnostic and treatment methods of epilepsy. As the epilepsy model animal, drug-induced epileptic animals produced by administration of kainic acid and kindling (method of repeating brain stimulation with electricity under a threshold value)-induced epileptic animals have heretofore been used.

However, the conventional model animals are not more than

animals in which convulsive seizure is forcibly induced and could not be used as true models for human epilepsy although they could be used as model animals of "convulsive seizure".

Meanwhile, thanks to recent progress in molecular biological studies, gene abnormality is being identified in several types of epilepsy. The inventors of this invention have found that there is a relationship between human chromosomal dominant nocturnal frontal lobe epilepsy and mutation of a neuron nicotinic acetylcholine receptor α4 subunit (CHRNA4) gene (Non-Patent Document 1) and that the mutation of CHRNA4 is specifically a substitution of Ser at position 284 by Leu (Non-Patent Document 2).

Non-Patent Document 1: Hirose, S. et al., Neurology 53: 1749-1753, 1999.
Non-Patent Document 2: Matsushima, N. et al., Epilepsy Res. 48: 181-186, 2002.

Disclosure of the Invention

The invention of this patent application has been accomplished in view of the problems of the conventional epilepsy model animals described above, and an object thereof is to provide a novel epilepsy model animal having gene abnormality (expression of mutant CHRNA4) homologous to human autosomal dominant nocturnal frontal lobe epilepsy and spontaneously presenting a somatic symptom identical to that of human autosomal dominant nocturnal frontal lobe epilepsy (epileptic seizure during sleep).

This patent application provides, as an invention for solving the

above-described problems, an epilepsy model animal (CHRNA4:S284L) developing spontaneous epileptic seizure during sleep, which is a nonhuman animal established by ontogenesis of a totipotent cell into which a polynucleotide encoding nonhuman mutant CHRNA4 is introduced and having said polynucleotide in its somatic chromosome, or a progeny of the nonhuman animal,

wherein said nonhuman mutant CHRNA4 has the corresponding mutation of human mutant CHRNA4 in which the 284th Ser of SEQ ID NO: 1 is substituted by Leu.

In a preferred mode of the epilepsy model animal (CHRNA4:S284L), the polynucleotide encoding the nonhuman mutant CHRNA4 is fused with a polynucleotide corresponding to a promoter region of a gene specifically expressing in cerebrum cortex and hippocampus.

Further, in another preferred mode of the epilepsy model animal (CHRNA4:S284L) of this invention, the nonhuman animal is a rat, and the polynucleotide encodes rat mutant CHRNA4 having the nucleotide sequence of SEQ ID NO: 2 in which the 865th c is substituted by t, and the 866th t is substituted by c.

In this invention, "polynucleotide" means a molecule obtainable by binding plural phosphoric esters (nucleotide ATP, GTP, CTP, and UTP or dATP, dGTP, dCTP, and dTTP) of a nucleotide in which purine or pyrimidine is β-N-glycoside-bound to sugar.

Also, "nonhuman mutant CHRNA4 corresponding to human mutant CHRNA4" means that Ser of a nonhuman CHRNA4 corresponding to Ser at position 284 of a human CHRNA4 (SEQ ID NO: 2) is substituted by Leu.

Other terms and concepts in this invention will be defined in detail in "Best Mode for Carrying out the Invention" and "Examples". Further, various technologies used for carrying out this invention other than those whose sources are indicated can be practiced by person skilled in the art easily and without fail based on known publications and the like. For example, the technologies of gene engineerings and molecular biology are described in Sambrook and Maniatis, in Molecular Cloning – A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989; Ausubel, F. M. et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, N. Y., 1995, and the like.

Brief Description of the Drawings

Fig. 1 is a diagram showing electroencephalogram recording during sleep of the epilepsy model animal of this invention. Shown in an upper part is the recording for 60 minutes; shown in a middle part is the recording of a cluster of spikes (for 15 seconds); and shown in a lower part is the recording of transition from the cluster of spikes to a slow wave complex, a gradual reduction in frequency, and a gradual increase in amplification (for 15 seconds).

Best Mode for Carrying out the Invention

It is possible to create the epilepsy model animal of this invention (CHRNA4:S284L) from various types of nonhuman mammals. For example, it is possible to create the epilepsy model animal from a mouse, a rat, a rabbit, a cat, a dog, a pig, a horse, a cow, and the like, and it is preferable to use the mouse or the rat in view of versatility and convenience as a

laboratory animal.

As the "polynucleotide encoding the nonhuman mutant CHRNA4" to be introduced, a mutant CHRNA4 polynucleotide corresponding to the subject animal species is used. For example, an amino acid sequence (SEQ ID NO: 5) of a mouse CHRNA4 and a sequence (SEQ ID NO: 4) of a polynucleotide (cDNA) encoding the amino acid sequence are known (GenBank/NM-015730), and Ser at position 286 of SEQ ID NO: 5 is corresponding to Ser at position 284 of the human CHRNA4. Therefore, it is possible to create a "polynucleotide encoding a mouse mutant CHRNA4" by substituting a Ser codon (tct) at positions 960 to 962 of SEQ ID NO: 4 by a Leu codon (ctt, ctc, cta, ctg, tta, or ttg). Also, an amino acid sequence (SEQ ID NO: 3) of a rat CHRNA4 and a sequence (SEQ ID NO: 2) of a polynucleotide (cDNA) encoding the amino acid sequence are known (GenBank/NM-024354), and Ser at position 286 of SEQ ID NO: 3 is corresponding to Ser at position 284 of the human CHRNA4. Therefore, it is possible to create a "polynucleotide encoding a rat mutant CHRNA4" by substituting a Ser codon (tct) at positions 865 to 867 of SEQ ID NO: 2 by a Leu codon (ctt, ctc, cta, ctg, tta, or ttg). It is possible to perform the nucleotide substitution by a method using a commercially available mutant introduction kit or a known method such as the mutant introduction PCR.

It is also preferable to introduce the polynucleotide encoding the nonhuman mutant CHRNA4 as a fusion polynucleotide of the polynucleotide and a polynucleotide corresponding to a promoter region of a gene specifically expressing in brain cortex and hippocampus. As the promoter region, it is possible to use a PDGF-β chain promoter and the like.

It is possible to create the epilepsy model animal (CHRNA4:S284L)

of this invention in accordance with a known transgenic animal creation method (for example, Proc. Natl. Acad. Scl. USA 77; 7380-7384, 1980). That is, it is possible to create a target transgenic animal by: introducing the polynucleotide (preferably the fused polynucleotide) into differentiating totipotent cells of the nonhuman animal; generating individuals from the cells; and selecting one into whose somatic genome the introduced polynucleotide is implanted from the individuals.

It is possible to use a fertilized egg or an early embryo as the differentiating totipotent cell into which the polynucleotide is introduced. As a method for gene introduction into a cultured cell, DNA physical injection (microinjection) is optimum in view of a high yield of transgenic animal individuals and efficiency of transmitting the introduced gene to the next generation. The fertilized eggs into which the gene is injected are transplanted to fallopian tube of a foster parent to develop individuals, and borne animals are raised by a foster parent. Then, a DNA is extracted from a part (tip of tail or the like) of the body of each of the animals to confirm existence of the introduced polynucleotide by Southern analysis or PCR. The individual (heterozygote) in which the existence of the introduced polynucleotide is confirmed is a founder (Founder: F0), and the introduced gene is transmitted to 50% of its descendants (F1). Further, it is possible to create individuals (F2) having the introduced gene in both of diploid chromosomes by crossing a male and a female of the F1 individuals.

As the method for introducing an exogenous gene to a chromosomal DNA of an animal individual, the knock in method using a known target gene recombinant method (Gene Targeting Method: Science 244: 1288-1292, 1989) is known. In the knock in method, an endogenous gene existing in a chromosomal DNA of an animal individual is perfectly

substituted by an exogenous gene. Therefore, the gene introduced-animal created by the knock in method does not produce the endogenous proteins but produces only exogenous (mutant) proteins homologous to the endogenous proteins. In contrast, the transgenic method used for the creation of the epilepsy model animal of this invention is capable of newly introducing the polynucleotide encoding the mutant CHRNA4 at an arbitrary position of a chromosomal DNA of an endogenous gene while maintaining the endogenous gene (CHRNA4 gene) in a normal state. Therefore, both of the normal CHRNA4 and the mutant CHRNA4 are produced in the epilepsy model animal of this invention. A neuron nicotinic acetylcholine receptor is a protein functioning as an ion channel (2 α subunits and 3 β subunits), and a channel function of such ion channel is modified when any one of the subunits is mutated. Therefore, the normal subunit and the mutant subunit may be expressed simultaneously, and the inventors of this invention have accomplished this invention based on the concept of considering the simultaneous expression of the normal subunit and the mutant subunit is more appropriate for a model animal presenting human epileptic seizure than the expression of the mutant subunit achieved by the knock in method.

The epilepsy model animal of this invention (CHRNA4:S284L) has the excellent characteristic of having spontaneous epileptic seizure during sleep as described in Examples, which is the same as that of human chromosomal dominant nocturnal epilepsy.

Examples

Hereinafter, the invention of this patent application will be described in more detail and specifically by way of examples, but the

invention of this patent application is not limited by the following examples.

(1) Method

(1-1) Creation of transgenic rats

PCR amplification of a rat fetus cDNA panel (Clontech, Palo Alto, CA) was performed for obtaining cDNAs of a rat Chrna4 and a rat Chrnb2, and then the cDNAs were subjected to sub cloning in a pCRTOPO II vector (Invitrogen, Carlsbad, CA). A nucleotide substitution (substitution of C at position 865 of SEQ ID NO: 2 by T, substitution of T at position 866 of SEQ ID NO: 2 by C; S286L) causing missense displacement corresponding to human nocturnal frontal lobe epilepsy was introduced in the Chrna4 cDNA by using Quickchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Into pSP64 Poly(A) vector (Promega, Madison, WI), cDNAs of a Wildtype Chrana4, a Chrnb2, and a mutant Chrna4 were introduced to create corresponding cRNAs, and the cRNAs were used for in vitro electrical biological study using xenopus oocytes. The mutant Chrna4 cDNA was inserted into an expression vector retaining a human PDGF-β chain promoter of pCI-neo vector (Promega). Integrity of each of the clones was confirmed by plural times of sequencing using a series of sequencing primers for each of the cloning steps. The vector retaining the mutant Chrna4 cDNA was cleaved by using Sna KI and NAe I, followed by purification of a chain fragment containing the mutant cDNA and the PDGF-β promoter. The fragment was injected into rat oocytes (SD) in Japan SLC, Inc. (Hamamatsu) to create transgenic rats.

(1-2) Confirmation of genotypes of transgenic rats

Tissue obtained by cutting a tale of each of the transgenic rats was digested overnight in a buffer solution [50 mM of Tris (pH 8), 10 mM of EDTA, 100 mM of sodium chloride, 1% (w/v) of SDS, and 50 mg/ml of

proteinase K (Sigma)] at 55°C. After an Rnase A treatment (100 μg/ml, at 37°C for 1 hour), ammonium acetate was added until a final concentration reached 2 M, followed by cooling, and then centrifugation was performed to precipitate a protein. A DNA in a supernatant was precipitated by using 0.6 vol of cool isopropanol, followed by washing with 70% ethanol. DNA pellets were dissolved into water at 4°C overnight. An amount of the DNA was evaluated at an optical density of 260 nm, and a part (50 ng) of the DNA was subjected to PCR amplification. The PCR product was subjected to sequencing with the use of an automatic sequence deciding apparatus (ABI 3100: Perkin Elmer Biosystems, Foster City, CA).

As a result, it was confirmed that each of the created transgenic rats had the introduced mutant Chrna4 cDNA in its somatic chromosome.

(1-3) Measurement of brain waves (EEG) of transgenic rats

Electroencephalography of each of the 8 to 10 weak-old transgenic rats was performed. After anesthesia using halothane (1.5% mixture of halothane and O_2 and N_2O), the rat was fixed to a brain fixing device, and then a Teflon (trademark) coating stainless electrode for electroencephalography was attached to the frontal lobe of each of right and left cerebral hemispheres (A = 3.2 mm, L = 0.8 mm from bregma), followed by fixing with dental cement. An indifferent electrode was embedded in an upper part of the cerebellum. Brain wave was measured by using a telemeter (Unimec Co., Tokyo, Japan) set to 0.1±3 kHz under the free motion condition from after 7 days from the attachment of electrodes. Brain wave analysis was performed also by using Chart for windows (Adinsruments, Sydney, Australia).

Results are as shown in Fig. 1. After a spindle wave characteristic of a nap, a cluster of spikes (middle part in Fig. 1) characteristic of the epileptic seizure was maintained for over ten seconds, followed by transition to a slow wave complex, a gradual reduction in frequency, and a

gradual increase in amplification (lower part in Fig. 1), whereby the epileptic seizure transitioned from the partial seizure to secondary general seizure.

As described in the foregoing, the created transgenic rat expressed mutant CHRNA4 and had the epileptic seizure during sleep. From the genotype of expressing the mutant CHRNA4 and the somatic symptom of epileptic seizure during sleep, it was confirmed that the transgenic rat was suitable as a model animal for human chromosomal dominant nocturnal frontal lobe epilepsy.

Industrial Applicability

As described in detail in the foregoing, the invention of this patent application provides an animal having gene abnormality homologous to human chromosomal dominant nocturnal frontal lobe epilepsy and having a somatic symptom identical to that of the human chromosomal dominant nocturnal frontal lobe epilepsy (epileptic seizure during sleep). This epilepsy model animal is remarkably useful for developments of diagnostic method and therapy for the human chromosomal dominant nocturnal frontal lobe epilepsy.